Passive Immunization with a Polyclonal Antiserum to the Hemoglobin Receptor of Haemophilus ducreyi Confers Protection against a Homologous Challenge in the Experimental Swine Model of Chancroid

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Haemophilus ducreyi, the etiologic agent of chancroid, has an obligate requirement for heme. Heme is acquired by H. ducreyi from its human host via TonB-dependent transporters expressed at its bacterial surface. Of 3 TonB-dependent transporters encoded in the genome of H. ducreyi, only the hemoglobin receptor, HgbA, is required to establish infection during the early stages of the experimental human model of chancroid. Active immunization with a native preparation of HgbA (nHgbA) confers complete protection in the experimental swine model of chancroid, using either Freund’s or monophosphoryl lipid A as adjuvants. To determine if transfer of anti-nHgbA serum is sufficient to confer protection, a passive immunization experiment using pooled nHgbA antiserum was conducted in the experimental swine model of chancroid. Pigs receiving this pooled nHgbA antiserum were protected from a homologous, but not a heterologous, challenge. Passively transferred polyclonal antibodies elicited to nHgbA bound the surface of H. ducreyi and partially blocked hemoglobin binding by nHgbA, but were not bactericidal. Taken together, these data suggest that the humoral immune response to the HgbA vaccine is protective against an H. ducreyi infection, possibly by preventing acquisition of the essential nutrient heme.

The sexually transmitted genital ulcer disease (GUD) chancroid is caused by the Gram-negative bacterium Haemophilus ducreyi (reviewed in references 9, 22, and 44). Although chancroid is currently considered rare in the United States (http://www.cdc.gov/std/stats08/other.htm), outbreaks of H. ducreyi infection occurred in large American cities throughout the 1980s and 1990s (10, 25). During these times, chancroid was endemic in sub-Saharan Africa, Asia, and the Caribbean (6, 44). It is difficult to assess the current epidemiology of chancroid because of syndromic management of GUDs and a lack of reporting and diagnostic tools. Some publications regarding the epidemiology of GUDs have described declining numbers of chancroid cases worldwide (8, 29, 40), while others have shown that H. ducreyi infections are still found in pockets throughout the world (2, 5, 15, 31). H. ducreyi has recently been shown to be the cause of lower limb cutaneous ulcers in patients from the South Pacific (24, 30, 46). Chancroid is also an important cofactor in the heterosexual transmission of the human immunodeficiency virus (HIV) (18, 32) and may have been particularly critical early in the HIV epidemic (38).

H. ducreyi is an obligate human pathogen. Unable to synthesize heme, H. ducreyi is thought to acquire this essential compound from its host by binding hemoglobin (Hb) or free heme using the TonB-dependent transporters (TBDTs) HgbA and TdhA, respectively (11, 21, 27, 41). Only 3 TBDTs are expressed by H. ducreyi: HgbA, an Hb receptor; TdhA, a heme receptor; TdX, which has not been assigned a function and is not expressed by all H. ducreyi strains (19). An isogenic hgbA mutant of prototypal strain 35000HP is avirulent in the human and rabbit experimental models of chancroid (3, 39), proving that HgbA is a virulence factor for H. ducreyi. Conversely, a double tdhAtdX mutant was fully virulent in the human experimental model (19), which suggests the following conclusions: (i) TdX and TdhA are not necessary for virulence in early steps of H. ducreyi infection in the experimental human model of chancroid; (ii) Hb is the most important source of heme for H. ducreyi; (iii) HgbA is the most important TBDT for acquisition of heme/iron by H. ducreyi.

By homology to other TBDTs, HgbA is thought to assume a pore-like structure in the outer membrane of H. ducreyi, with 22 β-strands, 11 putative surface-exposed loops, and a plug region present in the periplasm (B. Temple, unpublished data) (26). Using antisera from swine immunized with HgbA, our laboratory showed that loops 4, 5, and 7 of HgbA are immunogenic and that loop 4- and loop 5-specific antisera block Hb binding to HgbA (26). By generating single-loop deletion mutants of hgbA, we demonstrated that only deletion of loops 5 and 7 of HgbA substantially reduced Hb binding by HgbA. However, deletion of any loop of the HgbA protein prevented the use of Hb as a source of heme/iron by H. ducreyi (26). Taken together, these data indicate that a central domain of

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the primary amino acid sequence of HgbA is important for binding Hb by *H. ducreyi.*

Previous studies have shown that active immunization with a native preparation of HgbA purified from *H. ducreyi* prototypical class I strain 35000HP (HgbAII) protects against a homologous challenge in the experimental swine model of chancroid (1, 13). Protection was observed when using either Freund's adjuvant or an adjuvant approved for use in humans, mono-phosphoryl lipid A (MPL). Anti-nHgbAII antisera from both vaccine trials bound HgbA at the surface of *H. ducreyi* and partially blocked binding of Hb to nHgbAII. These *in vitro* correlates of protection suggest that the humoral immune response elicited to the HgbA vaccine may be protective. To obtain evidence that antisera developed to the HgbA vaccine may protect against an infectious challenge, we performed classic passive immunization studies with antisera elicited against nHgbAII in the experimental swine model of chancroid.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *H. ducreyi* strain 35000HP is the human-passaged variant (4) of wild-type isolate 35000 (14) and the prototypical strain for *H. ducreyi* class I strains (48). Strain FX547 is an isogenic hgbA deletion mutant of strain 35000HP (26), and FX548, an isogenic 35000HP strain in which the hgbB gene was replaced with hgbB of strain DMC111, a class II *H. ducreyi* strain (13). In this report, *H. ducreyi* strains 35000HP, FX547, and FX548 are designated 35000HP, 35000HPΔhgbA, and 35000HPΔhgbB, respectively. Other *H. ducreyi* strains used in this report include the 35000 HP isogenic delta hgbB mutant FX517 (12) and the isogenic monopΔ2 mutations (20), as well as the gmmA mutant, termed 35000.252 (7).

*H. ducreyi* strains were routinely grown on chocolate agar plates (CAPs) containing gonococal (GC) medium base (Difco, Detroit, MI) and 1% bovine Hb (Becton Dickinson, Sparks, MD) supplemented with 5% FetalPlex (Gemini Bio-Products, West Sacramento, CA) and 1% GGC (0.1% glucose, 0.001% glutamine, 0.026% cysteine) at 34.5°C in 5% CO2. For the purpose of nHgbAI purification and whole-cell binding enzyme-linked immunosorbent assays (ELISAs), *H. ducreyi* strains were cultured in low-heme GC broth (GCB; 1% GGC, 5% FetalPlex, and no addition of heme [1]).

**Animals.** A total of eight Yorkshire Cross (York) pigs (four pigs in each of two separate passive immunization experiments) were obtained at 3 weeks of age and housed at ambient temperature (20 to 25°C) in individual pens at the North Carolina State University (NCSU) School of Veterinary Medicine. Animals were given water and antibiotic-free high-protein feed ad libitum beginning 3 weeks prior to the start of and throughout the study. During inoculation and biopsy procedures, pigs were sedated with 2 mg of ketamine-HCl (Fort Dodge Labs, Des Moines, IA) and 2 mg of xylazine (Miles Laboratories, Shawnee Mission, KS) per kg of body weight, injected intramuscularly. At the time of biopsy, pigs generally weighed between 15 and 25 kg. The Institutional Animal Care and Use Committees (IACUC) at NCSU approved the methods and use of animals for these experiments.

**Preparation and passive immunization of the anti-nHgbAI polyclonal swine antisera.** A native preparation of the HgbA protein from class I *H. ducreyi* strain 35000HP (nHgbAII) was prepared as previously described from 12 liters of strain 35000HP grown in low-heme GCB (1). To ensure homogeneity of the nHgbAII preparation used for active immunization, the preparation was monitored by SDS-PAGE followed by Coomassie and silver staining (45), as well as Western blotting with monoclonal antibody 2C7 to assess for the presence of the major outer membrane proteins MOMP and OmpA2 (37). Based on these assays, the nHgbAII protein preparation was over 95% pure, with very little contamination with lipooligosaccharide (LOS) or MOMP/OmpA2 proteins (data not shown).

To generate the nHgbAII antisera, nHgbAII protein was sent to Covance (Custom Immunology Services, Denver, PA) for immunization of four York pigs. Since all published pig studies, including the passive challenge described herein, were done at NCSU, it is likely that the pigs used to develop anti-nHgbAII at Covance came from a family line unrelated to the animals used for passive immunization. This may explain the cross-reactivity seen in the antisera from these animals (see Fig. 3 and 4 below). At Covance, each pig received three immunizations of 250 μg of nHgbAII in complete (first immunization) and incomplete (second and third immunizations) Freund's adjuvant (Sigma-Aldrich, St. Louis, MO) at 3-week intervals, exactly as previously described (1). Three weeks after the last immunization, animals were exsanguinated, and serum was extracted from blood, aliquoted, and frozen at −20°C until being sent to our laboratory.

Two days prior to infusion, 50 ml of antisera from each of the 4 nHgbAI-immunized pigs was pooled into one preparation, filter sterilized, and divided into 50-ml aliquots. A pool of normal pig serum (NPS) was prepared in the same manner from sera obtained from three pigs from the NCSU Veterinary School which had previously been used for purposes other than infection (surgery) and were already scheduled for euthanasia. The aliquots were kept at 4°C until the day of the infusion. On the day of passive immunization, animals were sedated as described above. Fifty milliliters of blood was removed from each animal, and 50 ml of pooled nHgbAI antisera or NPS was passively administered through the brachiocephalic vein at the trunk by using a 60-ml syringe and a 1.5-in. 20-gauge needle. Depending on the size of the pigs (between 15 and 25 kg), and assuming a mean blood volume of 62.5 ml/kg (http://www.iauc.ucsf.edu/Proc/awSwineNorm.asp), the infused nHgbAI antisera accounted for 3 to 5% of the blood volume of passively immunized animals. Preparations of anti-nHgbAI and NPS were tested for the presence of endotoxin by using the end point chromogenic Limulus amebocyte assay from Lonza (catalog number 50-647U) following the manufacturer's instructions. All infused sera had endotoxin concentrations below 1 endotoxin unit/ml (data not shown).

For experiments described here, sera generated in the previously published active HgbA vaccine trial with Freund's adjuvant (1) were used as positive controls in many assays (see Fig. 3 to 6 below). These sera were purified from blood taken 3 weeks after the third immunization (prior to infection) from nHgbAI/Freund's-immunized animals (pigs number 6, 7, and 8; serum from pig 5 was not available).

**ELISA studies.** A direct ELISA (see Fig. 2, below) was used to evaluate the reactivities of individual and pooled nHgbAI antisera to purified nHgbAI, using changes to the protocol previously described (1, 13). An indirect ELISA, based on a kit from Bethyl Laboratories (catalog number E100-104; Montgomery, TX), was used to measure the quantity of nHgbAI-specific IgGs in the passively transferred antisera and the antisera from passively immunized animals. In this assay, wells coated either with nHgbAI or goat anti-pig antibodies (Abs) from the Bethyl kit were incubated with dilutions of the passively transferred antisera or antisera from animals passively immunized with anti-nHgbAI. Pig sera with known amounts of IgG were added to wells with anti-pig IgG to generate a standard curve. Wells were thereafter washed, incubated with anti-pig IgG conjugated to horseradish peroxidase (HRP), and developed as previously described (1, 13). The amount of nHgbAI-specific IgG in the pig antisera was determined by comparing the optical density obtained with the antisera to that of the standard curve (49). A whole-cell binding ELISA was also used to measure the reactivity of the antisera to HgbA expressed on the surface of intact *H. ducreyi* strains 35000HP, 35000HPΔhgbAII, 35000HPΔhgbB, and DMC111. A vacuum manifold was used to remove unbound components of the antisera, and Ab reactivity was determined using HRP-conjugated anti-pig IgGs, as previously described (1, 13). Procedures for *H. ducreyi* sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Total cellular proteins (from 2.5 × 107 CFU of *H. ducreyi* strains grown in low-heme GCB) were separated on a 4-to-12% gradient denaturing/reducing bis-Tris NuPAGE gel (Invitrogen, Carlsbad, CA) (150 constant volts) and transferred onto nitrocellulose for 2 h at 200 mA. The nitrocellulose was stained with Ponceau S for 5 min to monitor loading of the wells. After an overnight incubation in blocking solution (0.5% Tween 20 in phosphate-buffered saline [PBS]), four nitrocellulose membranes with the same bacterial antigens were concurrently processed and developed with the following antisera for 1 h at room temperature: anti-nHgbAII 1 (1), anti-nHgbAI 2 (current study), anti-recombinant HgbAII (rHgbAII) (28), all at 1:25,000, and anti-rD15 at 1:10,000 (42). After three 10-min washes with 0.05% Tween 20–PBS, blots were incubated with an alkaline-phosphatase (AP)-conjugated anti-pig or anti-rabbit secondary Ab for 1 h at room temperature. Blots were washed 3 more times before development with the AP chemiluminescence substrate Lumi-Phos WB (Thermo Scientific, Rockford, IL).

**Immunoprecipitation.** *H. ducreyi* strains were grown overnight under heme-limiting conditions to induce maximal expression of HgbAI (11). Cultures were centrifuged and pellets resuspended in GCB to an optical density at 600 nm (OD600) of 1.0 (approximately 5 × 10^8 CFU/ml). Ten microliters of serum was added to 1 ml of the bacterial suspension in a microcentrifuge tube and rocked at room temperature for 20 min. To remove unbound antibody and serum components, the suspension was centrifuged for 1 min at 14,000 rpm, the supernatant discarded, and the cell pellet washed with 1 ml GCB. The cell pellet was resuspended in 100 μl PBS, and 1 ml of 2% Zwittergent 3.14 (ZW 3.14) in TEN
buffer (50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, pH 8.0) was added to solubilize proteins. After incubation at 37°C with agitation for 1 h, the tube was centrifuged for 10 min at 14,000 rpm to remove insoluble debris. The supernatant (1.0 mL), containing ZW, 3.14-soluble proteins and Ab complexes, was moved to a new tube containing 25 μL of a 50% slurry of protein A/G-agarose beads (ExAlpha Biologicals, Shirley, MA). The tubes were incubated for 2 h to allow binding of Ab (and their respective bound cognate antigens) to protein A/G, centrifuged, and then washed thrice using 0.5% ZW, 3.14 in TEN. The agarose pellet was resuspended in 1.0 mL TEN and moved to a fresh tube and centrifuged, and the supernatant was discarded. Forty microliters of 1× Laemmli sample buffer lacking any reducing agents was added to the washed agarose, the tubes were boiled for 5 min at 95°C, and 15 μL was subjected to a 4- to-12% gradient SDS-PAGE gel and rapid Coomassie blue staining (20-min soak in 37 mL of 0.114% [wt/vol] Coomassie blue R-250 plus 20 mL of 0.0214% [wt/vol] Bismarck Brown in 40% methanol, 7% acetic acid).

Hb blocking assays. The ability of anti-nHgbA to block binding of digoxigenin-labeled Hb (DIG-Hb) to nHgbA was measured using two methods: an Hb-blocking ELISA, as previously described (13), and a whole-cell blocking ELISA. For both methods, 1 mg of bovine Hb (Sigma-Aldrich, St. Louis, MO) was labeled with DIG following the manufacturer’s instructions (Roche Diagnostics GmbH, Germany) and frozen at −80°C. In the Hb blocking ELISA, wells of a 96-well ELISA plate (Costar flat-bottom, high binding plate; catalog number 3590; Cambridge, MA) were incubated overnight with 100 ng of nHgbA, before blocking with 2% bovine serum albumin (BSA) in PBS. Purified anti-nHgbA IgG (20 μg of IgG purified using a protein A/G resin) was then added to each well and incubated for 30 min before addition of 400 ng of DIG-Hb in 1% BSA-PBS, allowing the incubation to continue for an additional hour. Wells were washed 3 times with 0.05% Tween 20 PBS, and then AP-conjugated anti-DIG (1:5,000, Roche Diagnostics, Indianapolis, IN) was added to each well and incubated for 1 h. After 3 more washes, the One-Step PNPP substrate (Pierce, Rockford, IL) was added to the wells, the plate incubated for 45 min, and optical density was measured at 405 nm by using the 1420 Victor2 multilabel reader (Perkin-Elmer, MA).

For the whole-cell Hb blocking assay, 1-ml aliquots of suspensions at an OD600 of 0.5 of H. ducreyi strains 35000HP, H. ducreyi II (13), and 35000HP hgbAII (nontype strains) (Indiana University, Bloomington, IN) grown in low-heme GCB, were mixed with 50, 100, or 250 μg of anti-nHgbA IgG (purified using protein A/G) for 30 min at room temperature. DIG-Hb (200 ng) was then added to the bacteria/IgG suspensions and incubated for another 30 min at room temperature. The cells were subsequently washed 3 times with GCB, moved to a new microcentrifuge tube, and washed one more time with PBS. The bacterial cell pellets were suspended in Laemmli sample buffer containing β-mercaptoethanol and subjected to SDS-PAGE (4 to 12% gradient gel) and Western blotting (1 h at 200 mA) with an AP-conjugated anti-DIG Ab (33500HP hgbAII, an isogenic strain of H. ducreyi 35000HP) that differs only in the expression of the heterologous HgbAII instead of HgbAI, were larger, raised, and inflamed (Fig. 1A, panel 1, right ear), similar to sites that developed in animals passively immunized with NPS after infection with either 35000HPhgbA1 or 35000HPhgbA2 (Fig. 1A, panel 2).

Microscopic analysis of H&E-stained biopsy specimens was consistent with microscopic observations. Sites infected with strain 35000HPhgbA1 in animals passively immunized with anti-nHgbA showed a low-level inflammatory infiltrate, and the dermis, epidermis, and basement membranes were intact (Fig. 1B, panel 3). Conversely, biopsy specimens from NPS-immunized animals infected with the homologous strain showed destruction of the dermis and epidermis and a massive inflammatory infiltrate (Fig. 1B, panel 5). A large influx of inflammatory cells and tissue destruction was also the hallmark of sites infected with the heterologous strain, regardless of the antisera used to passively immunize the animals (Fig. 1B, panels 4 and 6). H&E-stained biopsy sections were graded using the 1 to 5 grading system previously described (1, 13). Briefly, a score of 1 was assigned to healthy skin, while 5 characterized a fully developed ulcerative lesion. Animals passively immunized with anti-nHgbA and infected with the homologous strain had a mean lesion grade of 1.81 ± 1.1, compared to 4.42 ± 0.74 in animals infected with NPS (P < 0.001, t test). Conversely, there was not a statistically significant difference between the mean lesion grade of sites infected with the heterologous strain in nHgbA antisera-infused animals (4.1 ± 1.1) and animals that received NPS (3.75 ± 1; P = 0.567, t test).

Bacterial recovery was determined by culturing lesions taken from animals immunized with either anti-nHgbA or NPS. At the lower inoculum dose (106 CFU), animals that received the nHgbA1 antiserum were completely protected from a homologous challenge; we were unable to recover viable H. ducreyi from any of the 16 sites biopsied from 4 pigs (Table 1), compared to recovery of viable bacteria from all 12 sites in 3 pigs that received NPS (P < 0.001, Fisher’s exact test). At the higher inoculum dose (109 CFU), viable homologous H. ducreyi organisms were recovered from 3 out of 16 sites biopsied from the 4 animals immunized with the nHgbA1 antiserum (Table 1), compared to culture from all 12 lesions recovered in the 3 NPS-immunized animals (P < 0.001).

Statistics. Statistical analyses were performed using Sigma Stat (version 3.5, Systat Software, Chicago, IL).

RESULTS

Passive immunization with anti-nHgbA protected pigs from a homologous challenge. Two parameters were used to determine if passive immunization with anti-nHgbA protected against a challenge: tissue damage and bacterial recovery. Tissue damage was measured by determining the severity of the lesions at the macroscopic and microscopic levels. Macroscopic examination of the sites infected with the homologous strain 35000HPhgbA1 in animals passively immunized with anti-nHgbA revealed a few small, pink lesions, and in most cases, no lesion was visible, except for markings left by the applicator device (Fig. 1A, panel 1, left ear). Conversely, lesions resulting from infection with the heterologous strain 35000HPhgbA1, an isogenic strain of H. ducreyi 35000HP that differs only in the expression of the heterologous HgbA1 instead of HgbA2, were graded independently by two persons using the previously described histologic scale (1, 13, 35). Cohen’s kappa statistic for the two raters (κ = 0.544) indicated moderate agreement.
35000HPhbAI, expressing the heterologous HgbA protein was recovered from all sites on all animals, regardless of their immunization or the inoculum size (Table 1). Thus, there was complete protection from homologous infection at the lower challenge dose but no protection from a heterologous challenge was observed.

Potential mechanisms of protection of anti-nHgbAI. There are several mechanisms that may account for the protection observed in this passive immunization trial with anti-nHgbAI. From active immunization trials (1, 13), correlates of protection of the nHgbAI vaccine included cell surface binding as well as bactericidal activity and blocking of Hb binding; there was no indication that opsonophagocytosis was involved in the mechanism of protection of the HgbA vaccine (1). Sera from individual animals actively immunized with nHgbAI, the antisera used for passive transfer, as well as sera from passively immunized animals were therefore tested for reactivity to nHgbAI, binding to viable H. ducreyi, and bactericidal and Hb blocking activities.

Anti-nHgbAI binds purified nHgbAI. The reactivities of individual and pooled antisera to nHgbAI were first tested in a

TABLE 1. Recovery of H. ducreyi from immunized pigs

<table>
<thead>
<tr>
<th>Challenge strain</th>
<th>Expt no.</th>
<th>Pig</th>
<th>NPS immunization</th>
<th>Anti-nHgbAI immunization</th>
</tr>
</thead>
<tbody>
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<td>Class I strain, 35000HPhbAI</td>
<td>1</td>
<td>B</td>
<td>4 (100)</td>
<td>4 (100)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>E</td>
<td>4 (100)</td>
<td>4 (100)</td>
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<td></td>
<td></td>
<td>F</td>
<td>4 (100)</td>
<td>4 (100)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>12 (100) (A)</td>
<td>12 (100) (B)</td>
</tr>
<tr>
<td>Class II strain, 35000HPhbAII</td>
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<td>B</td>
<td>4 (100)</td>
<td>4 (100)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>E</td>
<td>4 (100)</td>
<td>4 (100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>4 (100)</td>
<td>4 (100)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>12 (100)</td>
<td>16 (100)</td>
</tr>
</tbody>
</table>

* Results followed by the same uppercase letter were significantly different: A and B, \( P < 0.001 \).
The activity of the nHgbA/I/Freund’s antisera was measured using an ELISA with nHgbA as coating antigen. Data are expressed as OD₄₅₀ readings, shown as means ± standard deviations obtained from at least 3 separate experiments. The left bar indicates the reactivity of the antisera from each donor pig actively immunized with nHgbA/I. The middle section of the graph (Exp. 1 and 2) shows the reactivity of the pooled antisera for each passive immunization experiment (pool) and that of the antisera from each animal after infusion with the pooled nHgbA/I antisera (C, D, G, and H). The right side of the graph shows the reactivity of NPS to purified nHgbA/I. Antisera were diluted 1:5,000.

Anti-nHgbA/I 2 exhibited cross-reactivity to H. ducreyi strain 35000HPΔhgbA in a whole-cell binding ELISA. To avoid confusion between antisera produced using Freund’s adjuvant, antisera from the study published in 2006 (1) were designated anti-nHgbA/I 1 and the antisera from the present study were designated anti-nHgbA/I 2. nHgbA/I antisera 1 was used in the various in vitro assays as a positive control.

In both active immunization trials with the HgbA vaccine, using either Freund’s or MPL adjuvants, reactivities of the antisera to HgbA at the surface of H. ducreyi correlated with protection (1, 13). To determine if nHgbA/I antisera 2 bound HgbA at the surface of intact, viable H. ducreyi cells, individual and pooled nHgbA/I antisera were subjected to a whole-cell binding ELISA. Reactivities of these different antisera to H. ducreyi strains 35000HPhgbA/I, 35000HPhgbA/I11, and DMC111 were compared to the reactivity of the antisera to the isogenic hgbA mutant 35000HPΔhgbA. There was high reactivity of all individual and pooled anti-nHgbA/I 2 to H. ducreyi strain 35000HPhgbA/I (Fig. 3A, right, and B, pool, black bars). However, anti-nHgbA/I 2 also exhibited high reactivities to strains 35000HPΔhgbA, 35000HPhgbA/I11, and DMC111 (Fig. 3A, right), which was not present in anti-nHgbA/I 1 (Fig. 3A, left).

To identify the antigen(s) targeted by the cross-reactive anti-nHgbA/I 2, Western blot assays were performed using total cellular protein from H. ducreyi strains 35000HPhgbA/I, 35000HPΔhgbA, 35000HPhgbA/I11, DMC111, and a panel of isogenic H. ducreyi mutants grown in low-heme GCB. Anti-nHgbA/I 1 showed little reactivity to any denatured bacterial component, including HgbA, in a Western blotting format (Fig. 4A), as previously described (1); however, anti-nHgbA/I 2 reacted with denatured HgbA (Fig. 4B). Anti-nHgbA/I 2 also showed minor reactivity to the major outer membrane proteins MOMP and OmpA2 (range, 31 to 45 kDa) (16). We were able to identify these bands as MOMP and OmpA2, as they were absent in the lanes containing isogenic mutants 35000HPΔmomp and 35000HPΔompA2 (Fig. 4B).

Anti-nHgbA/I 2 binds HgbA at the surface of H. ducreyi as measured in an immunoprecipitation assay. To ensure that anti-nHgbA/I 2 bound HgbA in a native conformation in the context of whole H. ducreyi cells and to identify putative targets of the cross-reactivity displayed in the whole-cell binding ELISA with these pooled antisera, anti-nHgbA/I 1 and 2 pools as well as control antisera were subjected to an immunoprecipitation assay (Fig. 5). Anti-nHgbA/I 2 precipitated only HgbA from H. ducreyi strain 35000HPhgbA/I grown in a low-heme culture. To confirm that this band was HgbA, these same immunoprecipitation samples were subjected to a Western blot assay using a rabbit polyclonal anti-rHgbAI antibody (28). The anti-rHgbAI Ab recognized a band only in those lanes where an HgbA band appeared in the Coomassie-stained gel (data not shown). Conversely, control lanes loaded with material from an immunoprecipitation assay using only protein A-agarose or agarose and IgG yielded either no band or bands that comigrated with the bands labeled IgG, respectively, in a Coomassie-stained SDS-PAGE gel (data not shown). Neither antisera precipitated nHgbA/I from strain 35000HPhgbA/I11, but both did so at a lower level in strain DMC111. Pooled antisera from naive pigs (NPS pool) did not precipitate any protein from any of the tested strains.

Anti-nHgbA/I 2 partially blocks binding of DIG-Hb to HgbA. Based on the data from the whole-cell ELISA and immunoprecipitation assays (Fig. 3 and 5, respectively) and the fact that anti-nHgbA/I 2 protected against a homologous challenge (Fig. 1), this second preparation of nHgbA/I antisera appears to bind HgbA in its native conformation. We therefore studied the biological activities of this antisera, including bactericidal activity and its capacity to block Hb binding to HgbA. No bactericidal activity was detected in any nHgbA/I antisera (data not shown). However, both pools of anti-nHgbA/I 2, as well as IgG purified from these antisera, partially blocked binding of DIG-Hb to purified nHgbA/I in an ELISA-type assay (Fig. 6A).
This activity was observed in ranges similar to that obtained with anti-nHgbA$_1$ 1 (69% inhibition compared to 64 to 66% inhibition for anti-nHgbA$_1$ 2). Anti-nHgbA$_2$ and IgG blocked DIG-Hb binding to HgbA significantly better than NPS or irrelevant anti-rDsrA antisera and IgG (Fig. 6A).

To determine whether anti-nHgbA$_2$ 2 could block Hb binding in the context of viable bacteria, we developed a whole-cell Hb blocking assay. As shown in Fig. 6B, addition of 50, 100, or 250 μg of anti-nHgbA$_2$ IgG significantly reduced the density of the Hb band on the Western blot by 15, 20, and 39%, respectively ($P < 0.026$ for 50 μg, $P = 0.004$ for 100 μg, and $P < 0.001$ for 250 μg of anti-nHgbA$_2$ 2 IgG; Mann-Whitney rank sum test), consistent with the ability of the antisera to block Hb binding to purified nHgbA$_1$ in the ELISA (Fig. 6A, 47 and 49% reduction for anti-nHgbA$_2$ 2 IgG experiments 1 and 2, respectively). In contrast, 250 μg of anti-nHgbA$_2$ 2 IgG did not significantly reduce binding of DIG-Hb to $H. ducreyi$ strains 35000HPg$_{HgbA_{II}}$ and DMC111 (4% reduction in band density, compared to results with no addition of anti-nHgbA$_1$ IgG), which express a class II HgbA protein on the bacterial surface.

**DISCUSSION**

Passive immunization with antisera elicited by the HgbA$_1$ vaccine protects against a homologous $H. ducreyi$ challenge in the experimental swine model of chancroid. Previous reports from our laboratory showed that a native preparation of HgbA, the Hb receptor of $H. ducreyi$, is a successful vaccine in the
Whether nHgbA is administered with Freund’s adjuvant or MPL, an adjuvant currently used in humans, the antisera elicited to the HgbA vaccine bound the surface of *H. ducreyi* and partially blocked Hb binding to HgbA. These data suggested that protection by the HgbA vaccine is Ab mediated. To test this hypothesis, we sought to determine if passive immunization of naïve swine with nHgbAI antiserum could protect against an infectious challenge with homologous and heterologous *H. ducreyi* strains. Infusion of pigs with anti-nHgbAI prevented infection with the homologous *H. ducreyi* strain 35000HP[nHgbAI] at an inoculum of 10<sup>3</sup> CFU. Thus, the humoral immune response elicited to the nHgbA vaccine protects against an infectious *H. ducreyi* challenge.

There was breakthrough infection at the higher inoculum dose (10<sup>4</sup> CFU) in 2 of 4 passively immunized animals. This may have been related to the concentration of nHgbA<sub>1</sub>-specific Abs present in the sera of infused pigs. Although the small sample size precluded a statistical analysis of correlations between antibody levels and passive protection, pigs with higher levels of nHgbA<sub>1</sub>-specific Abs were completely protected from challenge with either inoculum, while those with lower concentrations experienced breakthrough infections. These data suggest that the amount of nHgbA<sub>1</sub>-specific Abs is important for clearance of *H. ducreyi* in this animal model.

Anti-nHgbAI 2 displays the same *in vitro* correlates of protection as the ones identified in antisera from animals protected against a homologous *H. ducreyi* challenge. In 2 previous active immunization trials using nHgbA as a vaccine, antisera from nHgbA-vaccinated animals were tested for the ability to bind purified nHgbA and HgbA in the context of whole *H. ducreyi*, the ability to inhibit Hb binding to HgbA, and bactericidal and opsonophagocytic activities. In the first vaccination trial in which the nHgbA vaccine was administered with Freund’s adjuvant (anti-nHgbAI 1), the antisera from vaccinated animals bound purified nHgbA and HgbA at the surface of *H. ducreyi* strains, had modest bactericidal activity, and partially blocked DIG-Hb binding to nHgbAI, but lacked opsonophagocytic activity (1). In the second active immunization trial using MPL as the adjuvant, the antisera from animals immunized with nHgbA/MPL bound nHgbAI purified from *H. ducreyi* and HgbA in its native conformation, but with much less reactivity than anti-nHgbAI 1. Nevertheless, anti-nHgbAI/MPL blocked Hb binding to nHgbA in an ELISA to levels similar to that of the nHgbA<sub>1</sub>/Freund’s 1 antisera, but lacked bactericidal activity. Furthermore, the nHgbA vaccine administered with MPL
in the first trial, and none in the second, bactericidal activity does not appear to be necessary for protection.

The same in vitro assays described above were therefore used to assess the biological properties of individual and pooled anti-nHgbA1 antisera used for passive immunization experiments described here. These antisera bound purified nHgbA1 and HgbA1 on the surface of H. ducreyi and partially blocked Hb binding to HgbA1, again at levels similar to those observed for anti-nHgbA1 (Fig. 2, 5, and 6, respectively). However, there were some differences between the activities of anti-nHgbA1 2 and 1. First, there was higher cross-reactivity to the surface of the isogenic hgbA mutant strain 35000HP/NgbA1 by anti-nHgbA1 2. From Western blot assays, the anti-nHgbA1 2 bound denatured HgbA more than anti-nHgbA1 1 (Fig. 4A and B). Potential explanations for these differences include modification of the protein structure during preparation of the individual vaccines and genetic differences between swine herds; the animals used to generate anti-nHgbA1 2 came from a different farm and are likely distantly related to the animals that were previously used to conduct the HgbA vaccination trials. Anti-nHgbA1 2 also contained Abs that recognized the two major outer membrane proteins of H. ducreyi, MOMP and OmpA2 (Fig. 4B). There are a number of proteins from pig pathogens that have high homology to the major outer membrane proteins of H. ducreyi (23). Because these pathogens are early colonizers and infection by these pathogens is often endemic and mostly asymptomatic (P. Routh, personal communication), it is possible that one or more of the animals immunized with nHgbA1/Freund's vaccine may have been colonized with such cross-reacting bacteria. This would have contributed to the reactivity of the pooled anti-nHgbA1 2 to H. ducreyi MOMP and OmpA2. Western blot analysis of the nHgbA1 preparations used to generate anti-nHgbA1 2 with monoclonal antibody 2C7, which recognizes both MOMP and OmpA2 (37), revealed that MOMP and/or OmpA2 was present in the preparations; however, the amount was undetectable by Coomassie blue staining or silver staining (data not shown).

Antisera elicited to nHgbA1 only protect against a homologous challenge. H. ducreyi strains are grouped into classes, termed class I and class II, according to the expression of variant outer membrane determinants and the structure of LOS (33, 34, 36, 48). Although the amino acid sequences of some H. ducreyi surface determinants, such as DsrA and NcaA, differ widely between H. ducreyi strains belonging to different classes, the HgbA protein is highly conserved, with more than 95% identity between HgbA proteins in the two strain classes (26, 48). Most of these differences reside in the large immunodominant loop 4 of HgbA, which contains 17 different putatively surface-exposed amino acids out of a total of only 27 different residues between full-length HgbA proteins of different classes (950 total amino acids) (26). Because of this high identity between HgbA proteins of different groups, we were surprised to discover that the nHgbA1 vaccine did not protect against infection with H. ducreyi strain 35000HP expressing class II HgbA (13). The current data from passive immunization are consistent with these previous results. Pooled antisera elicited to the HgbA1 vaccine were only protective against infection with an H. ducreyi strain expressing HgbA1. Taken together, these data suggest that differences in a small number of immunogenic, variable residues in HgbA1
and HgbAII contribute to protection by an HgbA-based vaccine. Therefore, a bivalent HgbA vaccine may be necessary to prevent chancroid caused by both classes of H. ducreyi strains.

Another potential explanation for the lack of heterologous protection lies in the different exposure of the class II HgbA protein on the surface of H. ducreyi strains 35000HP and DMC111. The LOS of the class II strain DMC111 is truncated (48), and this smaller LOS structure may result in greater exposure of HgbA at the surface of class II H. ducreyi. However, because H. ducreyi class II strain DMC111 is noninfectious in the experimental swine model of infection, we were unable to examine the protective capacity of a class I antibody response against infection with a naturally occurring class II strain. It is therefore possible that natural class II strain infections may be protected by the class I vaccine. Further studies are needed to clarify this issue.

A possible mechanism of protection of the HgbA vaccine is nutritional immunity. Iron is required for the growth of most bacteria. However, iron in the host is sequestered from invading pathogens by several different proteins (47). Kochan used the term “nutritional immunity” to describe this process of “depletion by the host of iron essential for bacterial growth.” He associated this term with acquired immunity to relate it to the limitation of an essential nutrient by the host iron/heme-scavenging proteins (17). The idea of preventing a pathogen from acquiring an essential nutrient is decades old; however, this report, along with others from our laboratory (1, 13), are the first to suggest that vaccine-induced nutritional immunity can actually occur in the host. Our studies with the HgbA vaccine show that antisera elicited to an Hb receptor can partially prevent Hb binding to the Hb receptor itself, suggesting that nutritional immunity is possible (Fig. 6). Further experiments are under way to determine if anti-nHgbAII IgG can prevent bacterial growth.

In conclusion, we have shown in this report that passive immunization with pooled antisera from swine immunized with the Hb receptor of H. ducreyi protected naïve pigs against a homologous challenge in the experimental swine model of chancroid. Our results also suggest that the mechanism of protection of the HgbA vaccine may be nutritional immunity, since Abs elicited to the H. ducreyi Hb receptor were not bactericidal but partially prevented HgbA from binding its ligand, Hb.

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REFERENCES


